TOXICOLOGICAL EFFECTS OF MILITARY SMOKES AND OBSCURANTS ON AQUATIC THREATENED AND ENDANGERED SPECIES

J.C. Esarey and D.J. Soucek
Illinois Natural History Survey
Champaign, IL 61820

D.M. Cropek and T. Smith
U.S. Army Corps of Engineers
Champaign, IL 61822

1. INTRODUCTION

The U.S. Army must continually maintain a state of high readiness and alertness based on current geographical uncertainties. Preparation for adverse and unknown battlefield conditions requires military training activities using smokes and obscurants (S&O), and the need to effectively quantify the emissions resulting from S&O use and assess the potential health and environmental impact of these emissions has become a critical issue for the U.S. Army. Threatened and endangered species (TES), particularly fish and mussels, cohabit training areas where S&O are released; therefore, the impact of S&O on the vitality and survivability of aquatic TES must be ascertained.

Fog oil, graphite smoke, and colored signal smokes are among the most commonly used S&O. This paper details specific experiments within the larger framework of a multi-year project investigating the direct and indirect impacts of these S&O on two potential prey of TE fish, *Daphnia magna* (a filter feeding, planktonic crustacean) and *Chironomus tentans* (a benthic midge), using endpoints of mortality and fecundity. Characterization of the surface deposition and water column dissolution of these S&O is necessary to understanding potential effects on aquatic biota. While the study aims at development of methodology for testing more specific hypotheses regarding S&O impacts on aquatic TES, field measurement of relevant exposure concentrations (to be presented separately in Cropek et al. 2005) complimented by simultaneous field toxicity testing is an essential component. Acute field toxicity data obtained for *Daphnia magna* and *Chironomus tentans* exposed to fog oil, fog oil plus graphite, and colored smoke grenades are presented here and will serve to calibrate future controlled laboratory exposures.

2. MATERIALS AND METHODS

2.1 Study design

In May and August 2003, relevant life stages of *D. magna* and *C. tentans* were exposed at various intensities (determined by distance from release point) and durations of red, green, and yellow signal smokes, as well as to fog oil and a fog oil plus graphite at the Aberdeen Testing Grounds in Edgewood, MD. In the May experiments, exposure stations were placed at 5, 50, 100, 500, and 800 meters directly downwind of a release point (Table 1). A control station (C) was located 50 meters upwind. *D. magna* also was exposed to green (6 grenades) and yellow (16 grenades) smokes at a distance of 1 meter in May. In August, exposure distances were C, 5, 25, 50, 100, and 250 meters downwind (Table 2). These distances were determined to be more relevant following data collection and analysis from May exposures. All exposures were conducted in 500-ml, I-Chem glass screw cap jars with Teflon lid liners, with a synthetic moderately hard reconstituted water (MHRW) as a test medium (U.S. EPA 1993). Prior to exposure, 5 individuals of each test organism were placed into test jars and transported to the exposure stations. Four replicates for each species were placed at each exposure distance. Jar lids were removed immediately prior to S&O release and capped and transported to an onsite mobile laboratory immediately following exposure. Tested exposure durations ranged from 1-14 minutes for colored smoke grenades and 3-60 minutes for fog oil and fog oil plus graphite. Organisms were observed for 48 hours, and mortality was evaluated at the end of the test period. To correlate acute exposure results to S&O deposition, various collection media accompanied the test organisms at each distance. Results of the S&O deposition will be reported in a follow up document (Cropek et al. 2005).

Additionally, relevant life stages of *Pimephales promelas* (fathead minnow), *Rana pipiens* (northern leopard frog), *Etheostoma fonticola* (fountain darter), *Notropis topeka* (Topeka shiner), *Oncorhynchus mykiss* (Rainbow trout), *Potamogeton pectinatus* (sago pondweed), and *Pseudokirchneriella subcapitata* (previously *Raphidocelis subcapitata*, previously *Selenastrum capricornutum*, a green algae) were tested using endpoints of mortality, growth, and chlorophyll *a* loss when appropriate. While no significant impacts on these species were observed in May and August field

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 DEC 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Toxicological Effects Of Military Smokes And Obscurants On Aquatic Threatened And Endangered Species	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Illinois Natural History Survey Champaign, IL 61820; U.S. Army Corps of Engineers Champaign, IL 61822**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**6**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

exposures, they will be tested for sub-lethal endpoints under more controlled chamber conditions in future studies. These data will not be discussed further in this manuscript.

Table 1. *D. magna* and *C. tentans* exposures to smokes and obscurants. May 12-15, 2003.

Smoke or Obscurant	# Grenades or Duration	Exposure distance (meters)
Red Smoke	1	C, 5, 50, 100, 500, 800
Red Smoke	6	C, 5, 50, 100, 500, 800
Green Smoke	1	C, 5, 50, 100, 500, 800
Green Smoke	7	C, 1, 5, 50, 100, 500, 800
Yellow Smoke	1	C, 5, 50, 100, 500, 800
Yellow Smoke	7	C, 5, 50, 100, 500, 800
Yellow Smoke	16	C, 1
Fog Oil	3 minutes	C, 5, 50, 100, 500, 800
Fog Oil	18 minutes	C, 5, 50, 100, 500, 800
Fog Oil & Graphite	13:35 minutes	C, 50

Table 2. *D. magna* exposures to smokes and obscurants. August 4-6, 2003.

Smoke or Obscurant	Duration (minutes)	Exposure distance (meters)
Fog Oil	3	C, 5, 25, 50, 100, 250
Fog Oil	18	C, 5, 25, 50, 100, 250
Fog Oil	30	C, 5, 25, 50, 100, 250
Fog Oil	60	C, 5
Fog Oil & Graphite	3	C, 5, 25, 50, 100, 250
Fog Oil & Graphite	18	C, 5, 25, 50, 100, 250
Fog Oil & Graphite	60	C, 5, 25, 50, 100, 250
***D. magna* Fecundity**		
Fog Oil	18	C, 5, 25
Fog Oil & Graphite	18	C, 5

2.2 Culturing of test organisms

Daphnia magna neonates were cultured in the Illinois Natural History Survey (INHS) ecotoxicology laboratory according to U.S. EPA (1993) methods. Average (± standard deviation) pH, conductivity, alkalinity, and hardness for May 2003 culture and test water were 8.0 (±0.1), 278 (±6), 62 (±2) mg/L as CaCO3, 86 (±4) mg/L as CaCO3, respectively. Average (± standard deviation) water quality parameters for August culture and exposure water were 8.0 (±0.1), 289 (±5), 60 (±1) mg/L as CaCO3, 89 (±1) mg/L as CaCO3, respectively. Cultures were maintained at constant photoperiod (16L:8D) and temperature (25 ˚C). Prior to testing, organisms were fed a diet of *Pseudokirchneriella subcapitata* and Yeast-Cereal Leaves-Trout Chow (YCT) mixture daily at a rate of 1 ml each per 100 ml water. Four to five adult organisms were held in each 200-ml culture beaker; neonates were removed daily and held in 1-L beakers under the same conditions. Neonates were 5-7 days old upon initiation of 48-hour acute toxicity testing in May. Five-day-old neonates were used for 48-hour acute toxicity testing in August. Additionally, 10-day-old neonates were used in 96-hour fecundity tests in August exposures. *Chironomus tentans* individual and mass cultures were maintained in the INHS ecotoxicology laboratory according to U.S. EPA (1994) methods at constant photoperiod (16L:8D) and temperature (22 ˚C). Test waters were the same as described above. Prior to testing, organisms were fed a mixture of 20 g TetraMin® flake food and 20 g Kaytee ® Forti-Diet ® rabbit food (antibiotic-free) per 1 L of deionized water. Individual cultures were comprised of three to five egg cases and were held in 767-ml Rubbermaid containers. Each was fed at a daily rate of 3 ml mixture per 400 ml water. Aeration and feeding began upon hatching. Midges were 18-20 days old upon initiation of testing.

All organisms were transported to the field site in Aberdeen, MD, in 4-L, screw cap Rubbermaid containers. *C. tentans* required battery-powered aeration. On site, organisms were maintained under ambient light and air temperature conditions. Water temperatures ranged from 14-22 ˚C throughout the transport and testing period. Warmer August temperatures required a cooling mechanism to keep test waters in this temperature range. This was accomplished by nesting jars into trays of ice. Care was taken to not contaminate these samples with the ice water.

2.3 Acute toxicity testing field experiments

Mortality was recorded for each replicate at 24 and 48 hours. Since *D. magna* were found caught in a visible surface film created by the fog oil and fog oil plus graphite exposures, individuals caught in surface film were also recorded as sub-lethally affected at these times. In August experiments, *D. magna* fecundity was monitored at 24, 48, 72, and 96 hours. Adult mortality, number of adults caught in surface film, neonate production, and number of neonates caught in surface film were recorded at these intervals.

2.4 Data analysis

Mortality data were analyzed using Toxstat 3.5 and JMP IN 3.2.1. Data were tested for normal distribution (Shapiro-Wilks test) and for homogeneity of variance (Hartley's and Bartlett's tests). Normal and non-normal data (alpha=0.01) were examined using Fisher's Exact Test (p=.05). When a replicate had been eliminated due to contamination (Table 4) or failed homogeneity of variance tests, they were analyzed in JMP using Wilcoxon/Kruskal-Wallis Tests (Rank Sums). Normality and homogeneity of variance for sublethal endpoints were

determined by the above-described methods. Non-normal data with the same number of replicates were analyzed using Steel's Many-One Rank Test. Non-normal data with different number of replicates was analyzed in JMP IN by Wilcoxon/Kruskal-Wallis Tests (Rank Sums). Daphnia reproduction monitored in August trials was analyzed in JMP IN using Bonferroni t-test to compare total neonates and neonates per surviving adult. Surface catch was analyzed using Wilcoxon/ Kruskal-Wallis Tests in JMP IN.

3. RESULTS

3.1 *D. magna* mortality with colored smokes

For all red, green, and yellow colored smoke exposures, no significant mortality occurred for either *D. magna* or *C. tentans.* Control survivorship was above 95% for *D. magna* and between 65 and 80% for *C. tentans*. Investigation is currently under way to improve survivability of *C. tentans*. Significant ($p<0.05$) mortality was observed for *D. magna* replicates at 1m green (6 grenades) and yellow (16 grenades) smoke exposures. All organisms (n=20) died within 24 hours. Organisms were not exposed to red smoke at 1 meter.

3.2 *D. magna* mortality with fog oil and fog oil plus graphite

For both fog oil and fog oil plus graphite exposures, control survival was above 93% for *D. magna* and ranged from 70-95% for *C. tentans*. Mortality of *C. tentans* exposed to fog oil or fog oil plus graphite was not significantly different from that in controls in 48-hour acute field toxicity testing. However, *D. magna* experienced significant mortality at a distance of 5 meters from the release of the fog oil obscurant for the 3 and 18-minute durations in May (Table 3). Also, *D. magna* at 5 meters from 18, 30, and 60-minute fog oil exposures in August experienced significant mortality (Table 4a). No significant mortality was observed in the 48 hours following the 13.5-minute fog oil plus graphite exposure in May. However, August experiments yielded significant mortality of *D. magna* at 5 meters for the 18- and 60-minute fog oil plus graphite exposures (Table 4b).

Table 3: % *D. magna* mortality (n=20) 24- and 48-hours following exposure to fog oil in May 2003 (* indicates significantly different from the control (p=.05)).

	3 minutes		18 minutes	
dist..	24	48	24	48
-50	0	0	5	5
5	30*	85*	15	50*
50	0	0	0	10
100	0	0	0	10
500	0	0	0	0
800	0	0	0	0

Table 4: % *D. magna* mortality for (n=20) exposed to (a) fog oil or (b) fog oil plus graphite in August 2003 (* indicates significant difference from the control, ns = no sample, [a]n=15).

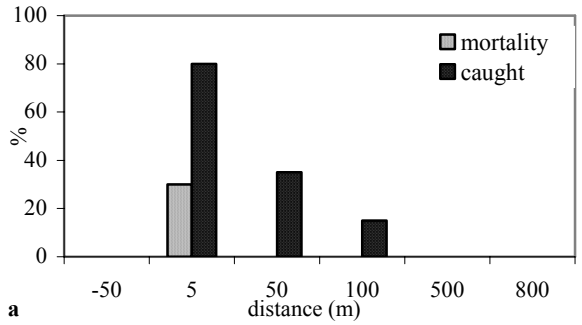
a: fog oil

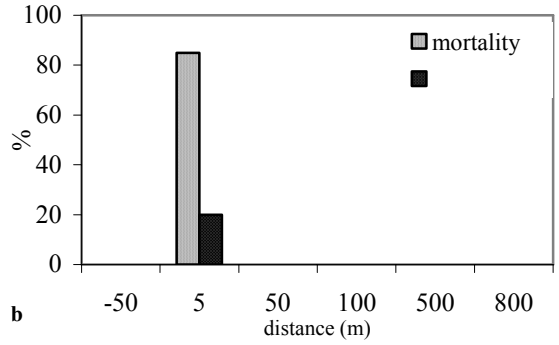
	3 minutes		18 minutes		30 minutes		60 minutes	
dist.	24	48	24	48	24	48	24	48
-50	0	0	0	0	0	0*	0	0
5	15	20	5	55*	5	65*	15	65*
25	10	10	5	5	15	15	ns	ns
50	5	10	10	15	0	0	ns	ns
100	15	20	0	5	5	15	ns	ns
250	0	0	0	0	20	15	ns	ns

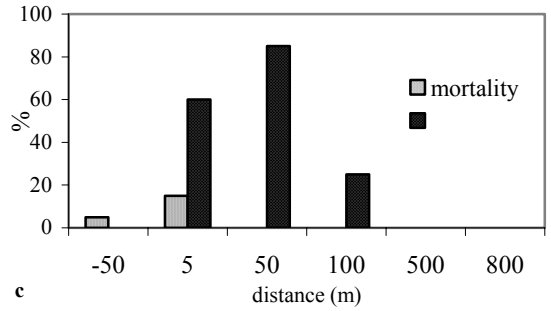
b: fog oil plus graphite

	3 minutes		18 minutes		30 minutes		60 minutes	
dist.	24	48	24	48	24	48	24	48
-50	0	0	0	7[a]	ns	ns	0	0
5	0	5	5	35*	ns	ns	30[a] *	55[a] *
25	0	0	5	25[a]	ns	ns	0	10
50	5	5	10	0	ns	ns	0	0
100	0	0	0	0	ns	ns	0	5
250	0	0	0	5	ns	ns	0	10

Sub-lethal effects for *D. magna* are presented in Figure 1a-d. In May, 80, 35, and 15% of neonates were caught in the surface film at exposure distances of 5, 50 and 100 meters, respectively, 24 hours after the 3 minute fog oil exposure (Fig 1a). However, only 20% of neonates in the 5-meter exposure were caught in the film at 48 hours (Fig 1b). This same pattern was observed at 24 and 48 hours following the 18-minute fog oil exposure in May. Sixty, 85, and 25 % of neonates at respective distances of 5, 50 and 100 meters from the release point were caught in a surface film (Fig. 1c). Again at 48 hours fewer neonates were caught, with only 5, 50, and 10% of the neonates at those respective distances (Fig. 1d). In the fog oil-graphite mixed obscurant, this phenomenon was not observed following the May exposure of 13.5 minutes.







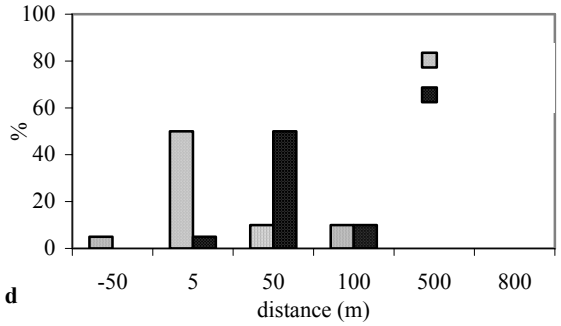


Figure 1a-d: Lethal and sublethal effects of fog oil on *Daphnia magna* neonates at various exposure distances in May field trials. (a) a 3-minute exposure after 24 hours and (b) 48 hours (c) an 18-minute exposure after 24 hours and (d) after 48 hours.

During August fog oil exposures of 3, 18, 30, and 60 minutes, *D. magna* were caught on the surface film at 5 meters after 24 and 48 hours (Table 5a). In the August 18 minute fog oil plus graphite exposure, 90% (24 hours) then 5% (48 hours) of *D. magna* were caught. Forty % (24 hours) and 13% (48 hours) of *D. magna* were observed caught following the 60 minute fog oil plus graphite exposure in August (Table 5b). For all obscurant exposures, *D. magna* was never stuck at the surface of any control replicates.

Table 5: % *D. magna* (n=20 unless indicated) caught in surface film 24-and 48-hours following exposure to (a) fog oil and (b) fog oil plus graphite in August 2003 (*indicates significantly different from the control (p=.05), [a] n=15).

a: fog oil

	3 minutes		18 minutes		30 minutes		60 minutes	
dist.	24	48	24	48	24	48	24	48
-50	0	0	0	0	0	0[a]	0	0
5	75*	75*	80*	35*	75*	25*	75*	30*
25	0	0	0	5	0	0		
50	0	0	10	0	0	0		
100	0	0	0	0	0	0		
250	0	0	0	0	0	0		

b: fog oil plus graphite

	3 minutes		18 minutes		30 minutes		60 minutes	
dist.	24	48	24	48	24	48	24	48
-50	0	0	0	0	ns	ns	0	0
5	25	0	90*	5	ns	ns	40*	13
25	0	0	0	0	ns	ns	20	0
50	0	0	0	0	ns	ns	0	0
100	0	0	0	0	ns	ns	20	0
250	0	0	0	0	ns	ns	0	0

3.3 *D. magna* fecundity with fog oil and fog oil plus graphite

Daphnia magna fecundity was tested following 18-minute fog oil and 18-minute fog oil plus graphite exposures in August (Table 2). No significant reductions were observed in adult mortality, number of neonates per surviving adult, or total number of neonates produced in fog oil experiments (Table 6a). However, fog oil plus graphite yielded significant adult mortality (25% of adults) at 5 meters (48 hours). Still no significant difference was seen in total neonate production or number of neonates per surviving adult (Table 6b). Significant numbers of adults and neonates were found caught in the surface film at 5 meters in both experiments. Seventy-five % of adults and 100% of neonates at both 24 and 48 hours were observed caught following the 18-minute fog oil exposure (Table 7a). Ninety % of adults and 100% of neonates were caught 24 hours following the 18-minute fog oil plus graphite exposure. Despite the substantial difference in the mean % adults and neonates caught at 48 hours, we did not find a statistical difference (Table 7b).

This is likely a result of low statistical power due to the fact that only two control replicates produced neonates.

Table 6: *D. magna* fecundity 24 and 48 hours following an 18-minute exposure to (a) fog oil and (b) fog oil plus graphite (*indicates significant mortality compared to the control (p=.05), $^{a}n_{adults}$=15, dist.= distance from release point in meters).

a: fog oil

	24 hrs		48 hrs		
	live		live		total
dist.	adults	neonates	adults	neonates	neonates
-50	15[a]	4	13[a]	11	15[a]
5	20	13	19	11	24
25	20	12	20	10	22

b: fog oil plus graphite

	24 hrs		48 hrs		
	live		live		total
dist.	adults	neonates	adults	neonates	neonates
-50	20	23	20	3	26
5	20	14	15*	16	30

Table 7: % *D. magna* adults and neonates caught in surface film 24 and 48 hours following an 18-minute exposure to (a) fog oil and (b) fog oil plus graphite (*indicates significant differences compared to the control (p=.05), $^{a}n_{adults}$=15, dist.= distance from release point in meters).

a: fog oil

	adults		neonates	
dist.	24	48	24	48
-50	0[a]	0[a]	0	0
5	75*	75*	100*	100*
25	0	0	0	0

b: fog oil plus graphite

	adults		neonates	
dist.	24	48	24	48
-50	0	0	0	0 (0/3)
5	90*	10	100*	50 (8/16)

4. Discussion

The intent of exposure trials was to determine if deposition of chemical constituents from actual releases of selected military S&O onto aquatic media produced measurable lethal or sub-lethal effects on aquatic organisms. These preliminary results should not be considered as definitive of test S&O aquatic toxicity. May and August field trials demonstrated that deposition of fog oil on aquatic surfaces produces both lethal and sublethal effects on *D. magna* in field exposures. Significant mortality was observed only relatively close to the release point; however, the sublethal effect of being physically trapped in the surface film was observed in substantial numbers up to 100 m away from the release point. The longer-term impacts of being trapped in the surface film are unknown at this time. This phenomenon was observed to a lesser extent in the fog oil plus graphite exposures.

While the chemical constituents of S&O prior to release are generally known, the chemical constituents released as a result of the combustion processes used in the generation of S&O are not well identified. Propensity to deposit, sensitivity to photolytic processes, volatility, and other factors serve to make prediction of deposition characteristics difficult. Separate but related experimental efforts, as a part of this overall effort, are underway to identify actual chemical compounds deposited on aquatic surfaces.

Climatologic conditions such as air temperature, light intensity, relative humidity, and wind direction and velocity may determine quantity and quality of S&O deposition and toxicity of deposited compounds. Exposure test starting times varied from 0600 hrs to 1700 hrs and ambient air temperatures varied from 54°F to 82°F. Variable wind direction and velocity confounded efforts to obtain uniform exposures and associated deposition. Some of the S&O appeared to loft or rise more than others. Whether this was related directly to wind, temperature, or chemical constituents, and/or other factors is uncertain. This lofting of the S&O had the observed effect of carrying it above the exposed jars that were at ground level. In contrast, some S&O stayed much closer to ground level and presumably resulted in increased exposure. Exposed jars were not always centered in the S&O plume due to shifting winds. Also, S&O plumes did not appear to be of uniform density as they proceeded down range. Presumably, chemical constituents and concentrations also varied with the varying density. Initial plans were to conduct exposure measurements to 1000 meters. While test range configuration allowed exposure measurement to approximately 800m, further exposure tests beyond 250m may be at times impractical because of the diffuse nature of the S&O plume beyond that distance and because of the difficulty in predicting wind direction and behavior down range.

While the varying ambient factors of field conditions pose a number of challenges to acute toxicity testing, our results show that acute toxicity is measurable in the field. Future experiments under controlled laboratory conditions will be important to understanding effects of ambient conditions on S&O deposition chemistry and will allow more detailed study of potential sub-lethal effects, such as fecundity and fitness, on aquatic life. Furthermore, we will be able to quantify dose dependent toxic effects of S&O.

This work will result in the development of products and approaches that (a) may be applied to chemical and other stressor effects on TES; (b) will provide both TES and surrogate species specific data on U.S. Army and U.S. Marine Corps specific chemical stressors; (c) will assist U.S. Army and other biologists and natural resources managers in the preparation of biological assessments in accordance with the Endangered Species Act (ESA); (d) will provide data to assist U.S. Army and other military service biologists and natural resources managers in the preparation and implementation of required Endangered Species Management Plans; (e) will assist the U.S. Army and other military services in the preparation of environmental assessments and environmental impact statements in accordance with the National Environmental Policy Act (NEPA) relative to the use, application, and effects of chemical stressors; and (f) will assist U.S. Army biologists, natural resources managers, and decision makers with risk assessment.

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of personnel at the Aberdeen Proving Ground, Aberdeen, MD and the Army Environmental Center. This work was supported by the Strategic Environmental Research and Development Program, project number CS-1332.

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